Combined Action of Nisin and Pediocin with Sodium Lactate, Citric Acid, Phytic Acid and Potassium Sorbate and EDTA in Reducing *Listeria monocytogenes* Population of Inoculated Fresh-cut Produce.

M. L. Bari*, D. O. Ukuku[1], T. Kawasaki, Y. Inatsu, K. Isshiki and S. Kawamoto

*National Food Research Institute, Food Hygiene Team, Kannondai-2-1-12, Tsukuba 305-8642, Japan

[1]Food Safety Intervention Technologies Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture†, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA

ABSTRACT

The inability of chlorine to completely inactivate human bacterial pathogens on whole and fresh-cut produce suggests the need for other antimicrobial washing treatments. Nisin (50 μg/ml) or pediocin (100 AU/ml) individually or in combination with sodium lactate (NaL) (2%), potassium sorbate (KS) (0.02%), phytic acid (0.02%), and citric acid (10 mM) were tested as sanitizer treatments for reducing population of *L. monocytogenes* on cabbage, broccoli, and mung bean sprouts. Cabbage, broccoli, and mung bean sprouts were inoculated with a five strain cocktail of *L. monocytogenes* at 4.61 log CFU/g, 4.34 log CFU/g, and 4.67 log CFU/g, respectively. Inoculated produce was left at room temperature (25°C) for up to 4 h before antimicrobial treatment. Washing treatments were applied to inoculated produce for 1 min and surviving bacterial populations were determined. When tested alone all compounds resulted in 2.20 to 4.35 $\log_{10}$ reductions of *L. monocytogenes* on mung bean, cabbage, and broccoli, respectively. The combination treatments nisin-phytic acid and nisin-pediocin-phytic acid caused significant ($p < 0.05$) reductions of *L. monocytogenes* on cabbage and broccoli but not on mung bean sprouts. Pediocin treatment alone or in combination with any of the organic acid tested was more effective in reducing *L. monocytogenes* populations than the nisin treatment alone. Although none of the combination treatments completely eliminated the pathogen on the produce, the results suggest that some of the treatments evaluated in this study, in particular, can be used to ensure the microbial quality of fresh-cut cabbage, broccoli, and mung bean sprouts.

INTRODUCTION

Over the past decade, the frequency of reported outbreaks of illnesses due to foodborne pathogens has increased (*10*). *Listeria monocytogenes* is a particular food safety concern because it is widespread in the environment (*2*), grows under refrigeration conditions (*8*), and is a frequent resident in certain food processing establishments (*4*). The microorganism has been isolated from soil, sewage sludge, vegetation, and water (*6*) and, therefore, has the potential to contaminate produce surfaces. Many vegetables, including bean sprouts, cabbage, cucumber, potatoes, and radishes have been found to be contaminated with *L. monocytogenes* (*1*). The pathogen has been reported to survive long term storage on leafy vegetables (*4*), has been responsible for numerous product recalls of salads (*18*), and was responsible for an outbreak of foodborne disease due to coleslaw prepared from contaminated raw cabbage (*1*). The

level of sanitation and the microbiological load are of primary importance to the quality, shelf stability, and safety of fresh produce (*3*). Chlorination of wash water up to 200 ppm is routinely applied to reduce microbial contamination in produce processing lines (*17*). However, the use of chlorine is of concern due to the potential formation of harmful by-products (*13*), and only approximately 2 to 3 log reductions of native microflora can be achieved (*15*). Thus, there is much interest in developing safer and more effective sanitizers.

Nisin and pediocin are produced by lactic acid bacteria that are often found on produce (*9*). There are several reports that nisin used in combination with a chelating agent exhibits a bactericidal effect towards both gram-positive and gram-negative bacteria (*7*). In the current study, the efficacy of nisin and pediocin treatments in combination with ethylenediaminetetraacetic acid (EDTA), citric acid, sodium lactate, potassium sorbate, and phytic acid in reducing populations of *L. monocytogenes* inoculated on fresh-cut produce was investigated.

MATERIALS AND METHODS

Bacterial Strains and Inoculum Preparation

A mixed bacterial cocktail containing five nalidixic acid resistant *Listeria monocytogenes* strains, ATCC 43256 (American Type Culture Collection, Manassas, VA) (Mexican-style cheese), ATCC 49594 (strain Scott A), JCM 7676 (Japan Collection of Microorganisms) (roast beef), JCM 7672 (salami sausage), and JCM 7671(lax ham) was used for the study. Individual cell cultures was prepared by inoculating from stock cultures stored at –80°C into 5 ml of Trypticase Soy Broth (TSB, Nissui, Japan) containing 50 μg of nalidixic acid per ml and incubating for 18 h at 37°C. Two successive loop cultures were made with a final transfer of 0.2 ml into 20 ml TSBY (TSB containing yeast extract) containing 50 μg/ml nalidixic acid and incubation at 36°C for 18 h under static conditions. Populations of individual cultures before mixing ranged from 1.63 to 2.11 x 10^8 CFU/ml as determined by plating serial dilutions on to Tryptic Soy Agar (TSA, Nissui) with incubation at 37°C for 24 h. The final bacterial concentration in the inoculum was approximately 6.40 to 6.65 x 10^7CFU/ml by plating on TSA containing nalidixic acid. The inoculum was maintained at 21±1°C and applied to fresh cut produce within 1 h of preparation.

Pediocin preparation, purification and antimicrobial bio-assay

Pediococcus acidilactici strain LET 01 isolated from uncured ham (provided by Dr. Takashi Sameshima, PRIMA Meat Packers Ltd., Ibaraki, Japan) was used for the production of pediocin. Pediocin preparation and purification was done as described by Cintas et al. (*5*). The spot on-lawn method as described by van Reenen et al. (*16*) was used to determine the antimicrobial activity of pediocin against *L. monocytogenes*. A clear inhibition zone of at least 2 mm in diameter was recorded as positive. One arbitrary unit (AU) of pediocin activity was defined as the reciprocal of the highest dilution that produced an inhibition zone of at least 2.0 mm in diameter.

Preparation of fresh produce and inoculation

Commercial cabbage, mung bean sprouts, and broccoli used in these experiments were purchased from a local supermarket and stored at refrigerated temperature (4°C) for up to 6 h before use. Broccoli and cabbage were cut into 3 × 3 cm size using a sterile knife on cutting board. Five hundred grams of mung bean sprouts, broccoli and cabbage were dipped in 2 L of the *L. monocytogenes* cocktail suspension (ca. 10^8 CFU/ml) for 1 min. After the inoculum was decanted, produce was placed separately

on a sterile perforated tray lined with four layers of cheesecloth and dried in a biosafety cabinet at room temperature (21±1°C) for 2 h.

Washing treatments

Final concentrations of the chemicals used alone or in combination were 0.02 M EDTA, 50 μg/ml nisin, 2 % NaL, 0.02 % KS, 0.02% Phy, 10 mM CA, 420 AU/ml pediocin. Two hours postinoculation, 25 g of each inoculated sprouts, fresh-cut broccoli, and cabbage pieces were placed in a Ziploc bag containing 50 ml of antimicrobial solutions, washed vigorously with agitation for 1 min. The produces were then transferred to a clean Stomacher bag (ELMEX Co Ltd., Tokyo, Japan).

Microbiological analyses

One hundred ml of 0.1% peptone water was added to Stomacher bags containing individual fresh-cut pieces, and the bag contents were pummeled for 60s in a Stomacher (ILU Instrument, Model CE-97, Barcelona, Spain) at medium speed. Serial decimal dilutions were prepared with 0.1% peptone water, and the diluted and undiluted samples were surface plated (0.1 ml, in duplicate) onto tryptose phosphate agar (TPAN) and modified oxford medium (MOXN), both supplemented with 50 μg/ml nalidixic acid for enumeration of *L. monocytogenes* with incubation at 37°C for 48 h. Representative presumptive colonies of *L. monocytogenes* were subjected to confirmation by use of API *Listeria* test kits (bioMeriux, Marcy l'Etiole, France). Plate Count Agar (PCA, BBL/Difco) and Potato Dextrose Agar (Nissui) with incubation at 30°C for 3 days were used for enumeration of mesophilic aerobes and yeasts and molds, respectively before and after the treatments. Desoxycholate agar (Nissui) with incubation at 37°C for 48 h was used for the enumeration of coliforms.

Statistical Analyses

All experiments were repeated three times, and duplicate samples were analyzed at each sampling time. Data were subjected to the Statistical Analysis System (SAS: SAS Institute, Cary, NC) for analysis of variance (ANOVA) and the Bonferroni LSD method to determine if there were significant differences ($p<0.05$) between mean values of the number of cells recovered after each treatment.

RESULTS AND DISCUSSION

Natural microflora of fresh-cut cabbage, broccoli, and mung bean sprouts.

The initial populations of natural microflora of the broccoli, cabbage, and mung bean sprouts determined after purchase varied among each produce (Figure 1). Broccoli had the least population of aerobic mesophilic bacteria followed by cabbage and mung bean sprouts. No *Listeria monocytogenes* was detected in the produce, and the level of yeasts and molds was below the level of detection (<1.0 CFU/g). The level of total coliforms determined in all of the produce tested was ca. 0.86 CFU/g for broccoli, 1.01 CFU/g for cabbage, and 1.87 CFU/g for mung bean sprouts. The presence of coliforms in the produce was indicative of sewage contamination and shows the level of microbiological quality of the produce. All antimicrobial treatments significantly ($p<0.05$) reduced the native microflora of the broccoli, cabbage and mung bean sprout samples compared to control samples. Yeast and mold, and total coliform of all treated fresh-cut produce were below detection (<1 CFU/g). (Data not shown)

Effect of antimicrobial treatments on inoculated fresh-cut produce

Levels of *L. monocytogenes* populations recovered from fresh-cut produce washed with water were not significantly ($p>0.05$) different from results of the untreated controls, with counts generally 0.10 to 0.60 log CFU/g lower with samples washed with water. Washing inoculated fresh-cut cabbage with nisin, and pediocin, EDTA, NaL, CA, Phy, and KS individually caused ~ 1.5 log CFU/g reductions in *L. monocytogenes*, and the reductions were not significantly ($P>0.05$) different, irrespective of the media used.

Figure 1.

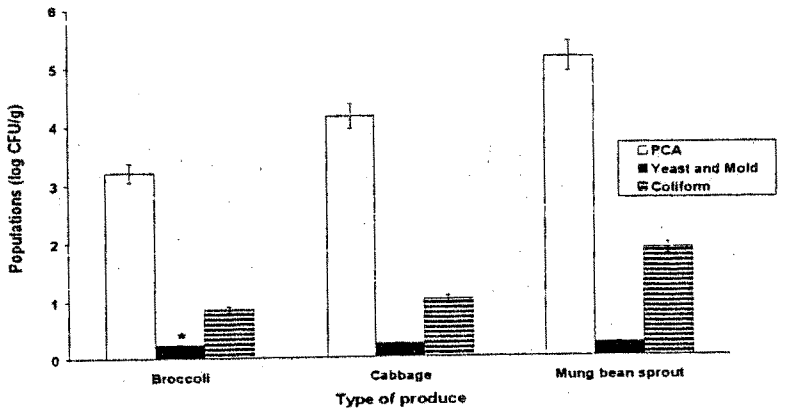

Figure 1. Initial populations of natural microflora of broccoli, cabbage, and mung bean sprouts before inoculation and treatments. Values are means ± S.D. of three experiments with duplicate determinations. * = Below detection limit of 1 CFU/g.

Population reductions of *L. monocytogenes* in fresh-cut produce washed with nisin or pediocin individually were not significantly ($p>0.05$) different than when the bacteriocins were combined with EDTA, NaL, CA, Phy, and KS (Table 1). Nisin treatments alone caused more reduction of *L. monocytogenes* in broccoli and cabbage than in mung bean sprouts. Also, the efficacy of nisin treatment alone is better than pediocin in reducing the populations of *L. monocytogenes* in fresh-cut broccoli and cabbage, but not in mung bean sprouts. Nisin treatment in combination with EDTA, lactate, sorbate, and Phy resulted in ~ 3.00 $\log_{10}$ CFU/g reduction of *L. monocytogenes*. Antimicrobial treatment containing nisin + Phy or nisin + pediocin + Phy combinations were the most effective in reducing the populations of inoculated *L. monocytogenes* on fresh-cut broccoli and cabbage but not on mung bean sprouts.

Treatment with pediocin, pediocin + CA, pediocin + EDTA, pediocin + lactate, pediocin + sorbate, and pediocin + Phy combinations resulted in approximately 2.0 $\log_{10}$ CFU/g reduction of *L. monocytogenes* on cabbage and broccoli, irrespective of the combination used (Table 1 and 2). All treatments were less effective in reducing the populations of *L. monocytogenes* on mung bean sprouts (Table 3) compared to broccoli or cabbage (Tables 1 and 2). The most effective antimicrobial treatment combination on mung bean sprouts was nisin + pediocin + phy, which caused a 2.31 log CFU/g reduction.

TABLE 1. Populations of *Listeria monocytogenes* recovered from cabbage following antimicrobial treatments[a].

Treatment	Population recovered (log CFU/g)		Reduction[b] (log CFU/g)
	MOXN	TPAN	
Control	4.55 ±0.15 A	4.61 ±0.19 A	-
Distilled water	3.51 ±0.05 B	3.75 ±0.07 B	0.86
Pediocin	2.30 ±0.30 C	2.67 ±0.15 C	1.94
Pediocin+ 10 mM CA	2.28 ±0.17 C	2.40 ±0.23 D	2.21
Pediocin+ 0.02% KS	2.31 ±0.09 C	2.65 ±0.12 C	1.96
Pediocin+ 0.02M EDTA	1.61 ±0.13 EF	2.17 ±0.21 E	2.44
Pediocin+ 2% NaL	2.43 ±0.10 C	2.57 ±0.11 CD	2.04
Pediocin+ 0.02% Phy	2.06 ±0.31 D	2.11 ±0.20 E	2.50
Nisin	1.59 ±0.10 EF	1.84 ±0.15 F	2.77
Nisin + 10 mM CA	1.70 ±0.12 E	2.14 ±0.21 E	2.47
Nisin + 0.02% KS	1.43 ±0.09 FG	2.18 ±0.10 E	2.43
Nisin + 0.02M EDTA	1.46 ±0.12 FG	1.67 ±0.13 F	2.94
Nisin + 2% NaL	1.36 ±0.10 G	1.39 ±0.14 G	3.22
Nisin + 0.02% Phy	0.24 ±0.09 H	0.26 ±0.10 I	4.35
Nisin + Pediocin+0.02% Phy	0.72 ±0.18 I	0.91 ±0.09 H	3.70

TABLE 2. Populations of *Listeria monocytogenes* recovered from broccoli following antimicrobial treatments[a].

Treatment	Population recovered (log CFU/g)		Reduction[b] (log CFU/g)
	MOXN	TPAN	
Control	4.29 ±0.12 A	4.32 ±0.08 A	-
Distilled water	3.65 ±0.08 B	3.80 ±0.10 B	0.48
Pediocin	2.77 ±0.16 CD	3.00 ±0.10 C	1.11
Pediocin+ 10 mM CA	1.90 ±0.09 G	1.95 ±0.13 G	2.35
Pediocin+ 0.02% KS	2.87 ±0.12 C	2.74 ±0.10 D	1.43
Pediocin+ 0.02M EDTA	2.40 ±0.07 F	2.42 ±0.09 F	1.90
Pediocin+ 2% NaL	2.76 ±0.18 CD	2.80 ±0.12 DE	1.53
Pediocin+ 0.02% Phy	2.50 ±0.18 EF	2.57 ±0.14 E	1.70
Nisin	1.67 ±0.15 H	1.73 ±0.10 H	2.55
Nisin + 10 mM CA	2.51 ±0.09 EF	2.57 ±0.12 E	1.70
Nisin + 0.02% KS	2.68 ±0.19 D	2.79 ±0.18 DE	1.55
Nisin + 0.02M EDTA	1.36 ±0.10 I	1.62 ±0.15 GH	2.47
Nisin + 2% NaL	2.64 ±0.24 DE	2.69 ±0.16 E	1.65
Nisin + 0.02% Phy	0.14 ±0.05 K	0.16 ±0.08 J	4.18
Nisin + Pediocin+0.02% Phy	0.31 ±0.03 J	0.44 ±0.07 I	3.90

[a]Mean values ± SD of three replicate experiments. Means in each column not followed by the same letter are significantly ($p<0.05$) different by the Bonferroni LSD means separation technique.
[b]within the same condition, $\log_{10}$ reduction compared with the number recovered from control on TPAN.

TABLE 3. Populations of *Listeria monocytogenes* recovered from mung bean sprout following antimicrobial treatments[a].

Treatment	Population recovered (log_{10} CFU/g)		Reduction[b]
	MOXN	TPAN	(log_{10} CFU/g)
Control	4.45 ±0.22 A	4.56 ±0.17 A	-
Distilled water	3.52 ±0.05 BC	3.77 ±0.09 BC	0.90
Pediocin	3.00 ±0.12 FG	3.05 ±0.23 GH	1.54
Pediocin+ 10 mM CA	2.96 ±0.10 G	3.08 ±0.16 H	1.59
Pediocin+ 0.02% KS	3.46 ±0.15 BCD	3.61 ±0.24 CD	1.06
Pediocin+ 0.02M EDTA	3.13 ±0.13 EFG	3.29±0.09 FGH	1.38
Pediocin+ 2% NaL	3.28 ±0.10 E	3.47 ±0.16 DE	1.20
Pediocin+ 0.02% Phy	3.27 ±0.17 E	3.31 ±0.13 EFG	1.36
Nisin	3.18 ±0.08 EF	3.36 ±0.21 EF	1.31
Nisin + 10 mM CA	3.29 ±0.12 DE	3.44 ±0.09 DE	1.23
Nisin + 0.02% KS	3.55 ±0.25 B	3.85 ±0.17 B	0.82
Nisin + 0.02M EDTA	3.01 ±0.16 FG	3.17 ±0.10 FGH	1.50
Nisin + 2% NaL	3.31 ±0.31 CDE	3.61 ±0.22 CD	1.06
Nisin + 0.02% Phy	2.95 ±0.15 FG	3.10 ±0.13 GH	1.57
Nisin + Pediocin+0.02% Phy	2.29 ±0.10 H	2.36 ±0.14 I	2.31

[a]Mean values ± SD of three replicate experiments. Means in each column not followed by the same letter are significantly ($p<0.05$) different by the Bonferroni LSD means separation technique.
[b]within the same condition, log_{10} reduction compared with the number recovered from control on TPAN.
Where, CA, Citric Acid; KS, Potassium Sorbate; NaL, Sodium Lactate; Phy, Phytic Acid.

The pH levels of all antimicrobial solutions used in this study is presented in Table 4. Sterile tap water (control) had the highest pH value. The pH of pediocin or nisin individually or in combination with CA, EDTA, lactate, sorbate, and Phy varied among the different solutions but was below 5.0. The pH of pediocin and nisin in combination with CA was the lowest, but this combination did not really give the highest reductions. Populations of *L. monocytogenes* recovered from inoculated treated fresh-cut produce were below 2.0 log unit. These reductions on fresh-cut vegetables are similar or greater than those achieved with aqueous chemical sanitizers or by washing with water. Foods that have been implicated in Listeria outbreaks generally contain more than 3.0 log units of bacteria per gram or milliliter (*14*). The combined treatments of nisin or pediocin with short chain organic acids tested in this study are consistent with the hurdle concept to ensure the safety of food (*11*). The reduced efficacy of nisin or pediocin alone or in combination with any of the organic acids tested on mung bean sprouts as compared to cabbage and broccoli may be attributed to the presence of inaccessible sites for the antimicrobial solutions to inactivate bacteria attached at to mung bean sprouts. Any of these treatment combinations can be combined with other control measures, such as modified atmosphere, water activity, pH, and temperature, to maximize protection from foodborne pathogens on fresh-cut produce (*12*).

TABLE 4. The pH of the treatment solutions.

Treatment solution	pH
Control (ddH_2O)	6.9 ± 0.01
Pediocin	3.4 ± 0.02
Pediocin+ 10 mM CA	1.8 ± 0.02
Pediocin+ 0.02% KS	5.0 ± 0.03
Pediocin+ 0.02M EDTA	2.1 ± 0.04
Pediocin+ 2% NaL	5.7 ± 0.03
Pediocin+ 0.02% Phy	2.6 ± 0.02
Nisin	2.8 ± 0.03
Nisin + 10 mM CA	1.8 ± 0.01
Nisin + 0.02% KS	4.8 ± 0.01
Nisin + 0.02M EDTA	2.0 ± 0.02
Nisin + 2% NaL	5.8 ± 0.01
Nisin + 0.02% Phy	2.5 ± 0.02
Nisin + Pediocin +0.02% Phy	2.3 ± 0.04

[a]Mean values ± standard deviation of three replicate experiments.
Where, CA, Citric Acid; KS, Potassium Sorbate; NaL, Sodium Lactate; Phy, Phytic Acid.

In conclusion, pediocin and nisin applications in combination with organic acids caused a significant reduction of native microflora and inoculated populations of *L. monocytogenes* on fresh produce. Nisin, is a natural antimicrobial compound and may provide a novel, environmentally safe alternative for control of bacterial contamination of produce. Although total inactivation of *L. monocytogenes* on produce surfaces tested was not achieved by the individual or the combined antimicrobial agents however, Nisin in combination with phytic acid significantly reduced the population of *L. monocytogenes* on cabbage and broccoli. Therefore, this combination could be useful in controlling the population of *L. monocytogenes* on cabbage and broccoli.

REFERENCES

1. Beuchat, L. R. 1996. *Listeria monocytogenes*: incidence on vegetables. *Food Control* 7:223-228.
2. Beuchat, R.L., and Brackett, R.E. 1991. Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. *Appl. Environ. Microbiol.* 57:1367-1371.
3. Brackett, R.E. 1992. Shelf stability and safety of fresh produce as influenced by sanitation and disinfection. *J. Food Prot.* 55, 808-814.
4. Carlin, F., and Nguyen-The, C. 1996. Fate of *Listeria monocytogenes* on four types of minimally processed green salads. *Lett. Appl. Microbiol.* 18:222-226.
5. Cintas L M, J. M. Rodriguez, M. F. Fernandez, K. Sletten, I.F. Nes, P. E. Hernandez, and H. Holo. 1995.Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Appl Environ Microbiol.* 61:2643-8.

6. Cox, L. J., Kleiss, T., Cordier, J. L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, and R., Siebenga. 1989. *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiol.* 6:49-61.
7. Cutter, C.N., and Siragusa, G.R. 1995. Population reductions of gram negative pathogens following treatments with nisin and chelators under various conditions. *J. Food Prot.* 58:977-983.
8. Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55:476-511.
9. Fleming, H.P., J.L. Etchells, and R.N. Costilow. 1975. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Appl. Microbiol.* 30:1040-1042.
10. Foods and Drug Administration/Center for Food Safety and Applied Nutrition. 2001. Direct food substances affirmed as generally recognized as safe. p. 462–464.Title 21 of the Code of Federal Regulations—Food and Drugs, chapter 1, part 184. Food and Drug Administration, Department of Health and Human Services, Washington, D.C.
11. Leistner, L. 1992. Food preservation by combined methods. *Food Res. Int.* 25:151-158.
12. Leverentz, B., W. J. Janisiewicz, and W. S. Conway. 2002. Biological control of minimally processed fruits and vegetables. p. 319-332. In J. S. Novak, G. M. Sapers, and V. K. Juneja (ed.). Microbial safety of minimally processed foods. CRC Press, Boca Raton, Fla.
13. Richardson, S.D., Thurston, Jr., A.D., Caughran, T.V., Collette, T.W., Patterson, K.S., and Lykins Jr., B.W. 1998. Chemical by-products of chlorine and alternative disinfectants. *Food Technol. 52.* 58-61.
14. Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65:709-725.
15. Ukuku, D.O. and W.F. Fett. 2002. Effectiveness of chlorine and nisin-EDTA treatments of whole melons and fresh-cut pieces for reducing native microflora and extending shelf life. *J. Food Safety* 22:231-253.
16. Van Reenen, C.A., L. M. Dicks, and M. L. Chikindas. 1998. Isolation, purification and partial characterization of planttaricin 423, a bacteriocins produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* 84: 1131-1137.
17. Wei, C. I., D.L. Cook, and J.R. Kirk. 1985. Use of chlorine compounds in the food industry. *Food Technol.* 39: 107-115.
18. Wong, S., Street, D., Delgado, S.I., and Klontz, K.C. 2000. Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration. *J. Food Prot. 63.* 1113-1116.